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The amino acid sequence of the 115kD subunit of the Vacuolar-type ATPase proton pump has been analyzed and predicted to have six membrane-spanning domains. PCR primers have been designed and used to amplify DNA sequences coding for five of six targeted nonmembrane-spanningdomains. The amplified DNA sequences have been purified and ligated into the pGEX-2T expression vector for generation of GST fusion proteins,to be usedto test the specificity of OSW-2, a monoclonal antibody targeting the 115kD subunit, andto be used to generate antibodies targeting the nontransmembrane-spanning domains of the subunit.

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FOREWORD

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Introduction

Vacuolar type H^+ ATPases (V-ATPase) are heteromultimeric proteins found in all eukaryotic cells, and have been highly conserved among divergent species. V-ATPases couple hydrolysis of ATP to the translocation of protons across a membrane and are largely responsible for the acidification of intracellular vesicles and membrane-bound compartments. In addition, some cell types express V-ATPases at the plasma membrane, where they may play a role in pH regulation or the acidification of extracellular compartments.

The pump complex is comprised of "V_1" and "V_0" regions (by analogy with the related F_1F_0 H^+ ATPases). V0 is a hydrophobic, integral membrane region comprised of a hexamer of 16kD subunits and single copies of 115, 45, and 39kD peptides. The 16kD hexamer is thought to form the proton "pore'. V1 contains a hydrophillic "head", composed of three copies each of A (70kD) and B (60kD) subunits, and an intervening "stalk", with single copies of C (41kD), D (34kD), and E (33kD) subunits. Both the A and B subunits have ATP binding sites, with catalysis thought to occur on the A subunit, and the ATP binding properties of the B subunit proposed to be regulatory. The specific function of other subunits is unknown. Although ATP catalysis of both C and E subunits, these and other subunits are thought to mediate V1-V0 association and/or play other regulatory roles.

The existence of different isoforms of various pump subunits has been demonstrated, and it has been proposed that these alternative isoforms may result in cell- or organelle-specific targeting or specificity of activity of VATPases in different tissue types or subcellular compartments. For example, osteoclasts, which constituitively express VATPase at the plasma membrane (for

acidification of extracellular bone resorptive compartments), have been shown to express different isoforms of A and B (Chatterjee et al 1992) and 115 kD (Li et al 1996) subunits. These isoforms may provide for specific targeting and regulation of plasma membrane VATPase activity distinct from that of "housekeeping" VATPase activity in endocytic compartments, Golgi, lysozomes, etc. Alternative splicing has been shown to give rise to different isoforms of the A and 115kD (Peng et al 1994) subunits in a tissue-specific manner, and distinct genes encode different isoforms of the B and 115kDsubunits. Thus far, cell types expressing unique subunit isoforms are those in which altered VATPase activity has been demonstrated or hypothesized i.e. extracellular compartment acidification by osteoclasts or renal epithelia, synaptic vesicle acidification in brain. In contrast, the classical, ubiquitous isoforms are present in most cell types. Demonstration that distinct genes code for alternative forms of the 115kD homolog in yeast (Manolson et al 1994) suggests that this subunit may play a role in targeting or regulation of the pump within a single cell. Other subunits with unknown function- C, D, E, 39kD, 45kD, etc., may also be involved in regulation, but well-developed models of VATPase regulation are currently lacking.

A number of cell types express VATPases at the plasma membrane. In these cells, including osteoclasts, macrophages, renal epithelia and neural cells, the plasmalemmal VATPase expression is important to some specialized cell function. Osteoclasts use the pump to acidify an extracellular bone resorptive compartment during skeletal development. Macrophages, which may recruit up to 50% of plasma membrane during phagocytosis, constituitively express VATPase at the plasma membrane, likely in order to facillitate rapid phagosomal acidification. Apical plasma membrane of renal intercalating cells is highly enriched in VATPase for H+ export into the urine, and in neurons rapid acidification of plasma membrane-derived synaptic vesicles is neccesary for effective concentration of neurotransmitter.

In addition to the aforementioned cell types, a large number of cancerous cell lines exhibit plasma membrane VATPase activity, as demonstrated by a bafilomycin-sensitive ability to recover from an acid load in absence of Na^+ and HCO_3^- (Martinez-Zaguillen et al 1992). The significance of this activity in cancerous cells is not clear, but a number of possibilities exist. VATPases are not usually thought to be significantly involved in cytosolic pH maintenence, as VATPase activity is low with respect to the Na^+-H^+ exchanger (NHE). However, VATPases clearly have the potential to significantly affect cytosolic pH, especially at alkaline values , where NHE activity is diminished. Proliferative and cancerous cells generally maintain a higher cytosolic pH than do normal cells, even within tumors where localized extracellular pH may be very acidic. Plasmalemmal VATPase activity could be significant in allowing cells to maintain this exagerrated pH gradient, sustaining cytosolic conditions conducive to growth, and even selecting for these cells in acidic environs.

Our attempts to visualize VATPase at the plasma membrane in cells expressing this activity via immunocytochemistry have been inconsistent at best. This suggests the possibility that this activity may be dynamic, i.e. involving rapid cycling of VATPase-containing vesicles with the plasma membrane. Such a mechanism would allow for exocytotic removal of protons from the cell, while not requiring extensive, static residence of the VATPase in the plasma membrane itself.

As was described in last year's report and the revised Statement of Work, our aim is to generate fusion proteins using PCR-amplified sequences of the 115kD subunit. These fusion proteins will be used to determine the specificity of OSW-2, a monoclonal antibody targeting the 115kD subunit. These fusion proteins will also be used to generate antibodies against domains of the 115kD subunit., These antibodies will be used to a) epitope map the 115kD subunit relative to its orientation in the membrane, b) determine by Western analysis if the 115kD protein is upreguated in response to acidic environment in a number of breast cancer cell lines, c) determine by antibody uptake

experiments the effect of blocking endocytosis on plasmalemmal VATPase activity. As our model predicts that the VATPase activity seen in cancer cell lines expressing this activity is due to rapid cycling of VATPase-containing vesicles, we predict that interfence with endocytosis will block plasmalemmal VATPase activity in these cells, and may reverse the drug resistance that correlates with functional plasma membrane VATPase activity in a number of breast cancer cell lines.

Body

Materials and Methods

Sequence Analysis The amino acid sequence of the human 115kD protein was analyzed by the standard Kyle-Doolittle algorithm. The resulting hydropathy plot indicated six likely membrane-spanning domains.

PCR PCR primers were designed and generated to amplify six of the seven nonmembrane-spanning domains. Each pair of primers were designed to have a 5' BamH-1 cut site and a 3' EcoR-1 cut site for subsequent ligation into the pGEX-2T expression vector. The full-length cloned cDNA of the 115kD subunit was used as a template, and PCR conditions for each target sequence optimized over many trials. The ExpandTM High Fidelity PCR Kit (Boehringer Mannheim) was used in all PCR reactions.

DNA Extraction and Restriction Digestion PCR products were electrophoresed in 1.5% agarose in TAE gels, stained with ethidium bromide, and the product bands excised from the gel. The Dna was purified from the agarose using the Prep-A-Gene DNA Isolation Kit (Bio-Rad). Eluted DNA was double digested with EcoR-1 and BamH-1 in Buffer B (all Gibco Life Technologies). The restriction digested DNA was again electrophoresed and purified from agarose gels, and purified for subsequent ligation.

Ligation and Transformation The pGEX-2T expression vector (Pharmacia Biotech, designed for production of GST fusion proteins) was double digested with EcoR-1 and BamH-1 and treated with Alkaline Phosphatase (Boehringer Mannheim). The cut plasmid was combined with cut PCR product at a DNA concentration ratio of 1 plasmid : 5 PCR product. The ligation was performed using T-4 DNA Ligase and Ligation Buffer (Boehringer Mannheim) at 15C overnight.

Transformation DH5a competent *E.coli* (Gibco Life Technologies) were transformed using standard protocol. The bacteria were plated onto ampicillin + agar plates and incubated overnight. Colonies were picked and used to inoculate ampicillin + Luria broth. 2ml cultures were grown overnight, miniprepped, and the recovered DNA

double digested with EcoR-1 and BamH-1. The cut DNA was run on agarose gels to determine the presence of appropriate PCR product insert.

Results and Discussion

Analysis of the predicted amino acid sequence of the 115kD subunit of the human VATPase proton pump strongly suggests the presence of six membrane spanning domains. Figure 1 shows a cartoon of the predicted topology of this protein relative to the membrane. In this figure, cytosolic and extracellular compartments are not delineated, as the orientation of the protein is not known (this is a question I hope to shed light on, see Statement of Work). Figure 1 also indicates the size of the amino acid sequences that define the different nonmembrane-spanning domains, as well as the amino acids in the sequence that are coded for by the DNA amplified via PCR. One such domain was not targeted, as it was deemed too small to amplify, purify, and use for ligation and efficient antibody generation.

After many trials, I successfully generated single band PCR products of the appropriate size for five of the six targeted sequences. Examples of these are shown in the extended Figure 2. As is common when attempting to generate PCR products, there was much trial and error in attempting to optimize reaction conditions, with some targeted sequences being more difficult to successfully amplify than others. The volume of failed attempts precludes me from including them all in this report, but the figures that are included, showing successful product generation, also contain examples of failed attempts. In addition, despite innumerable attempts, I was never able to successfully amplify target #2, and have reconciled with working with five of the six originally designated target sequences.

The map of the pGEX2-T expression vector to be used in the generation of GST fusion proteins is included as Figure 3. The use of two different restriction enzymes in cutting the plasmid and the use of complementing restriction sites in the PCR primers removes the question of directionality in analyzing the inserts after ligation. However, a number of researchers have informed me, through personal communication, that inserts

are difficult to visualize upon attempts to excise the inserts by restriction endonucleases subsequent to ligation and transformation. I do not know the explanation for this, but have seen it in this work. Figure 4 shows the attempt to visualize the appropriate sized inserts on an ethidium bromide stained gel. This is miniprepped DNA recovered from bacteria successfully transformed with the PCR-pGEX construct, after digestion with the appropriate restriction enzymes. Despite the definite presence of appropriately sized inserts in some lanes, they are very difficult to see, in contrast to inserts cut from plasmids in general. Thus, the only sure way (other than sequencing, which we hope to avoid at this time for financial reasons) to determine the correctness of those constructs not yet confirmed by digestion and electrophoresis is through induction of the GST fusion protein expression, and identification of expressed protein of the predicted size. This is the work that is immediately at hand as this report is being written.

Recommendations/ Statement of Work

The work on this project is behind schedule relative to the outline in the Statement of Work. This is due to both intrinsic and extrinsic factors. The time spent generating PCR products of the correct size was far more than I had anticipated. In addition, we exhausted the supply of OSW-2 antibody, which was a gift of Dr. Satoshi Sato (Japan). At our request, Dr. Sato sent us the hybridoma cell line producing this antibody. What I imagined to be a technically simple matter of isolating the antibody from the supernatant media these cells are grown in and purifying it via ammonium sulfate precipitation and Protein A incubation (as Dr.Sato described), instead turned into a very time consuming and frustrating exercise for myself. This work was subsequently given over to a technical assistant in our lab and, despite continued difficulties, she has recently had some success in this endeavor. As noted in the S.O.W., I had intended to use the 115 cDNA to determine the chromosomal location of the gene encoding this subunit by FISH. Upon submission of the cDNA probe to the commercial FISH laboratory here at the Arizona Cancer Center, I was informed that they are unable to accomplish this work with any probe less than 5kb in size. This aim has therefore been removed, as the information is

not central to this work. I have also proposed to determine the effects, if any, on mRNA and protein expression of this subunit by Northern and Western analyses, respectively. I currently have two breast cancer cell lines, MCF-10A parental and h-ras-transfected MCF-10T, each growing at both pH 6.8 and pH 7.4. Northern analysis of these cells using a 1.1kb fragment of the 115 cDNA will be performed in the coming days. Western analysis of these same cell lines will be performed upon generation of antibodies, which should be completed in the coming two to three months.

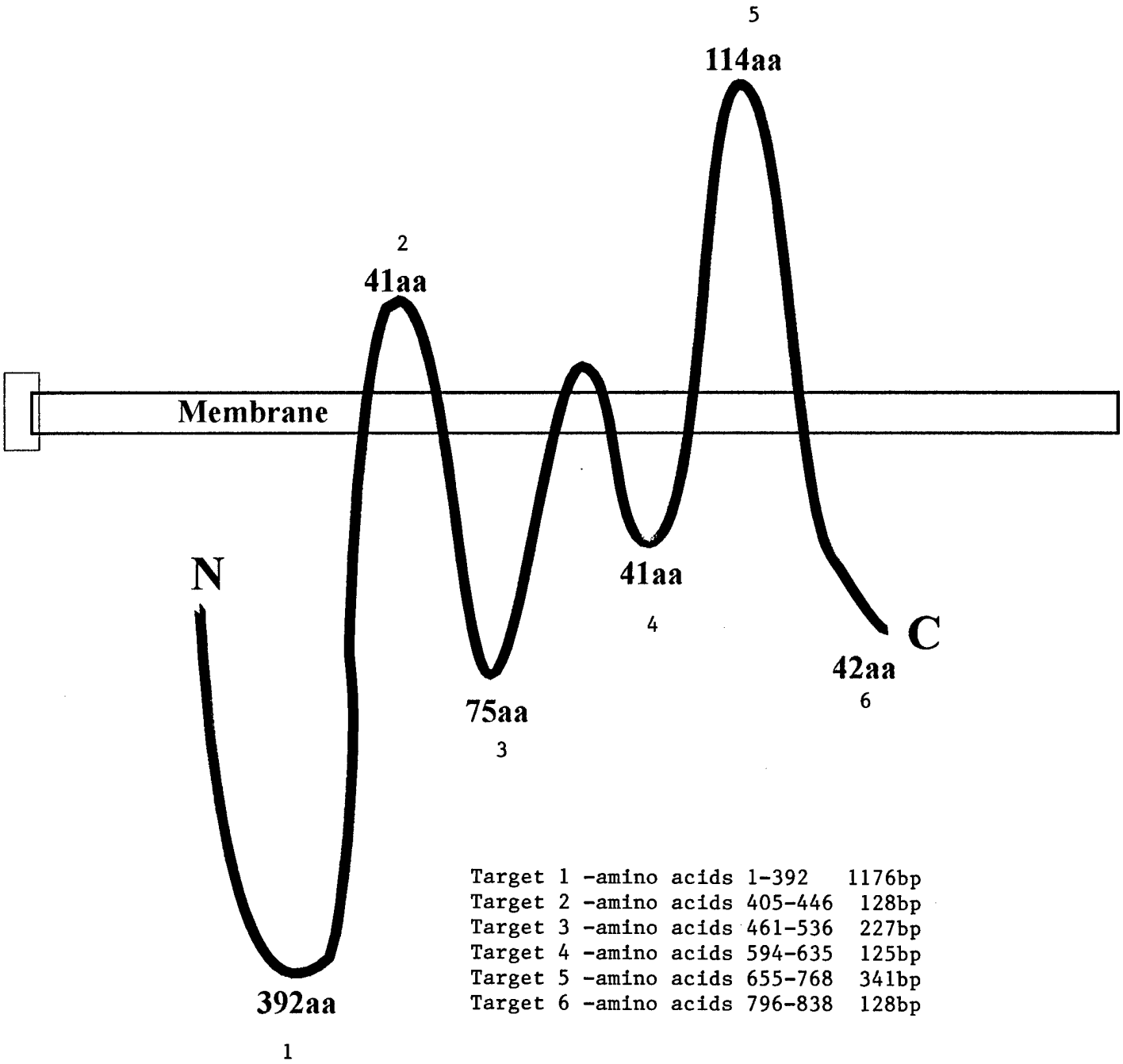
In addition to the aforementioned problems and experimental delays, time has also been lost to some extrinsic factors. I spent a number of weeks away from the bench preparing for (and passing) my oral preliminary examinations. In addition, our lab moved in the past months to the new Cancer Center here at the University of Arizona. It was a welcome move to a superior lab, but also cost significant time. Lastly, I've been assigned to work with a number of undergraduates working on unrelated projects in our lab. I understand that this is a normal circumstance for a graduate student, but one I had not faced before. None of this is intended as excuse for being behind my projected schedule, simply an explanation of circumstances.

Conclusions

As noted in the preceeding pages, the important results have been in progress made towards the generation of reagents (antibodies) necessary for getting results in subsequent experiments. This is a somewhat linear proposal of work, and as such, a significant amount of work must be done before meaningful experiments can be undertaken. To this end, I've determined sequences within the 115 cDNA that code for nontransmembrane-spanning domains. These are the domains I intend to use in generation of GST fusion proteins and subsequent antibody generation. I have designed PCR primers targeting these sequences and have successfully amplified these sequences. The amplified DNA products have been ligated into the pGEX expression vector and the resultant construct used to successfully transform competent bacteria. Induction of fusion protein expression is underway at the time of this writing.

Despite being behind the schedule I set forth in my S.O.W., I am pleased with the progress that has been made in the last two to three months, in contrast to much of the preceeding time. I expect to generate the antibodies necessary for much of my proposed work during the remainder of the summer, and anticipate completing all of the work proposed in the S.O.W. within a year's time.

FIGURE 1. Predicted topology of the 115kD subunit in the membrane.



Target 1	-amino acids 1-392	1176bp
Target 2	-amino acids 405-446	128bp
Target 3	-amino acids 461-536	227bp
Target 4	-amino acids 594-635	125bp
Target 5	-amino acids 655-768	341bp
Target 6	-amino acids 796-838	128bp

Figure 2. PCR products

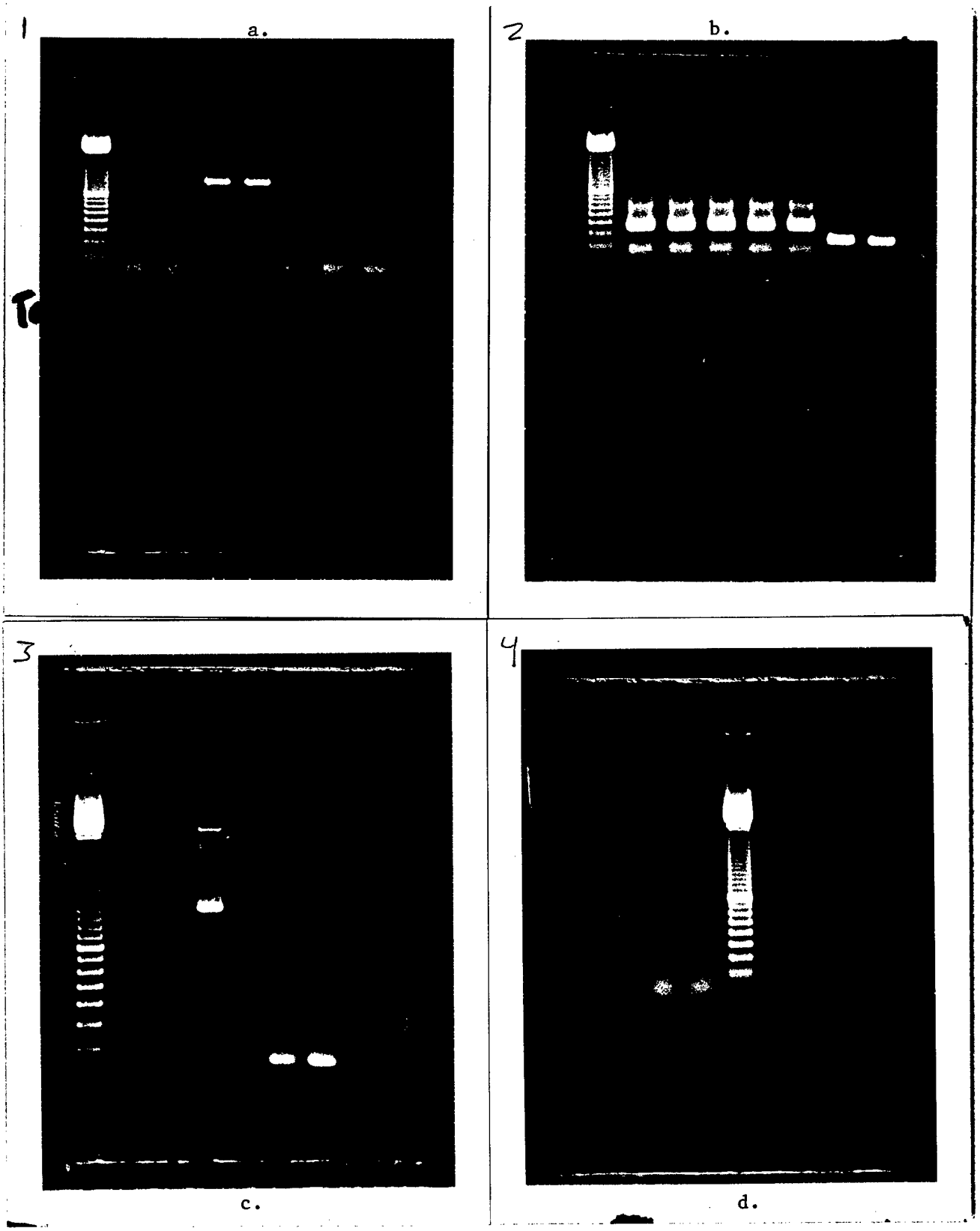
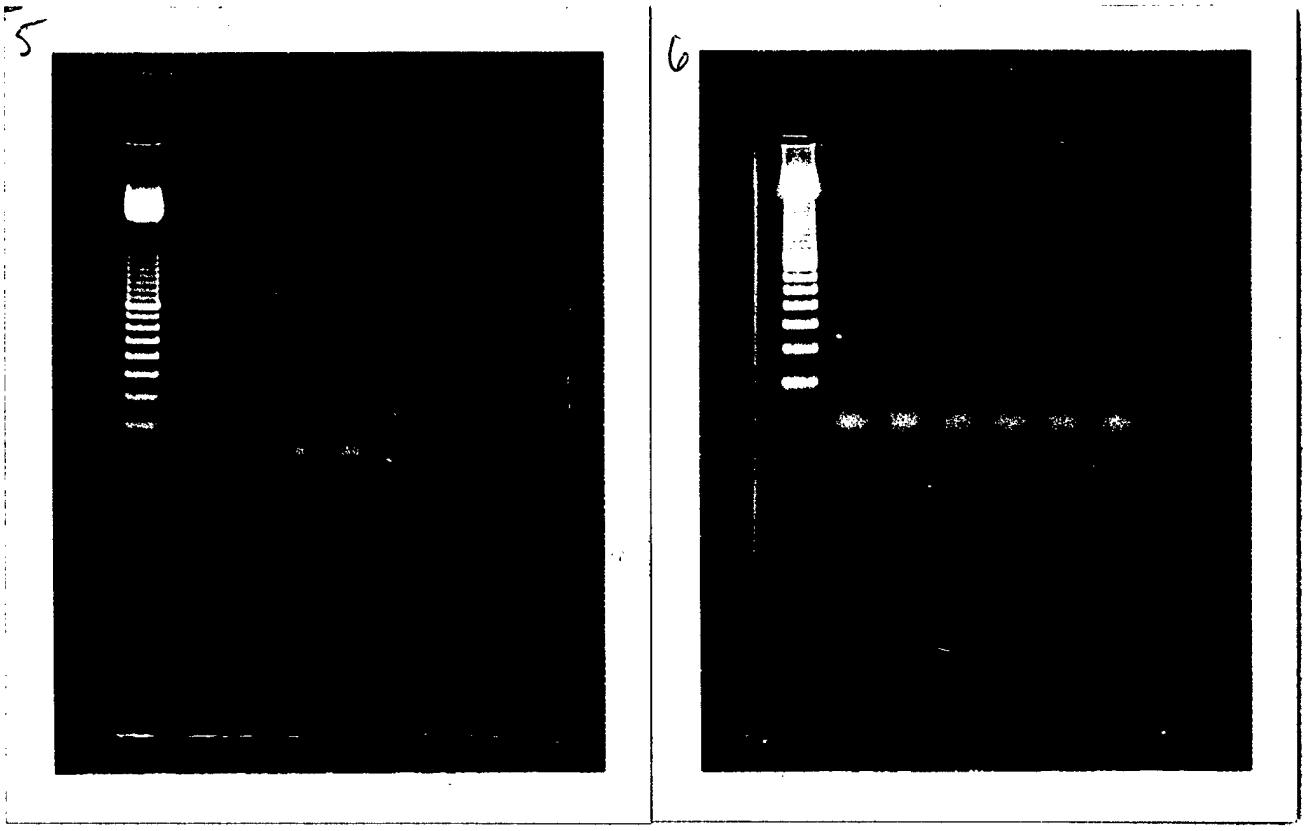


Figure 2 (con't) PCR products



e. f.

Figure 2. a) Lane 1-100bp ladder, lanes 4 and5-1176bp PCR product #1, other lanes -failed reactions
b) Lane 1-100bp ladder, lanes 2-6 -247bp PCR product #3, other lanes -failed reactions
c) Lane 1-100bp ladder, lane 3-1176bp PCR product #1, lanes 5-7 -125bp PCR product #4, other lanes- failed reactions
d) Lanes 1 and 2 -341bp PCR product #5, lane 3- 100bp ladder
e) Lane 1-100bp ladder, lanes 6-7 -128bp PCR product #6, other lanes- failed reactions
f) One of innumerable experiments where no products were generated

Figure 3. pGEX map.

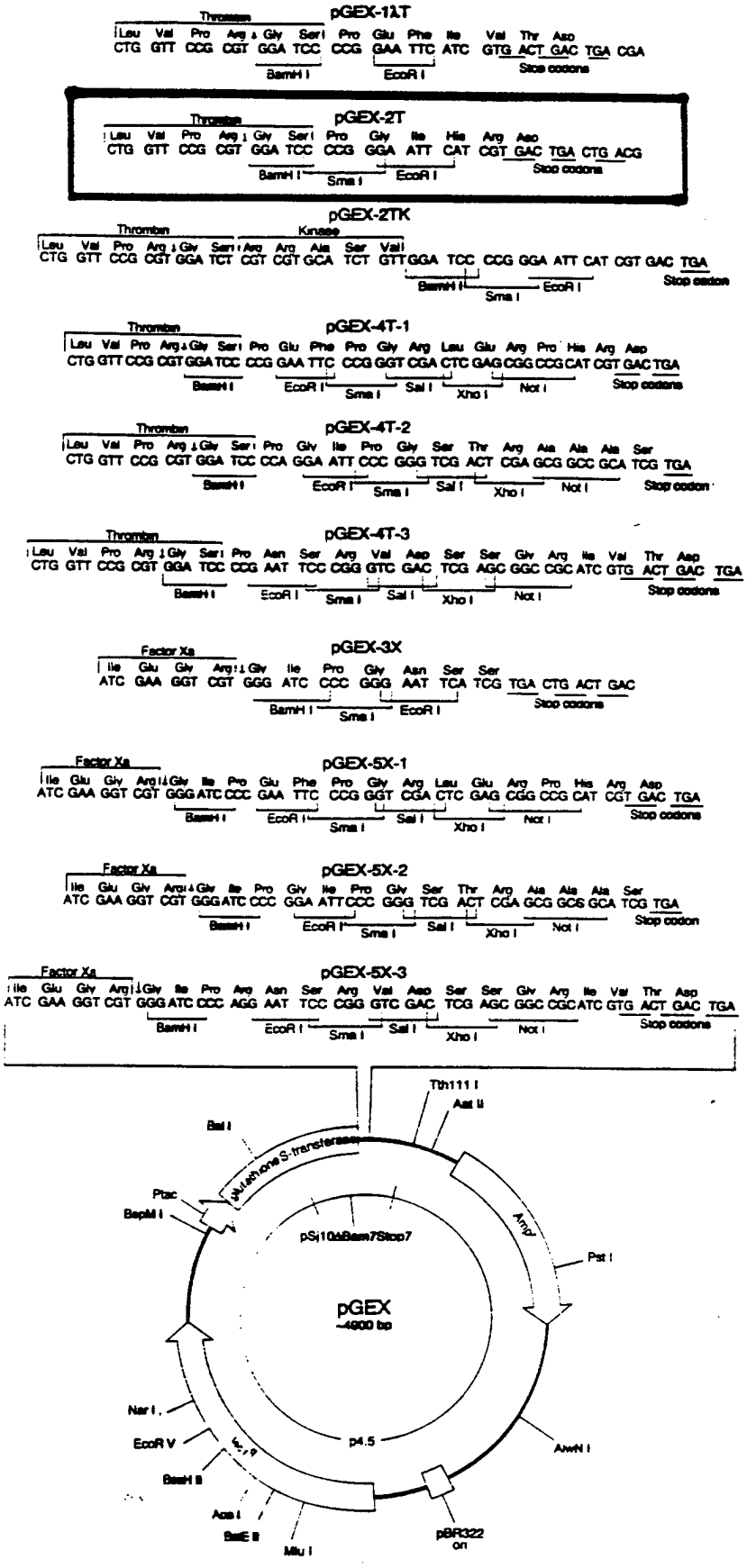


Figure 4. Miniprepped pGEX-PCR product constucts after restriction digestion

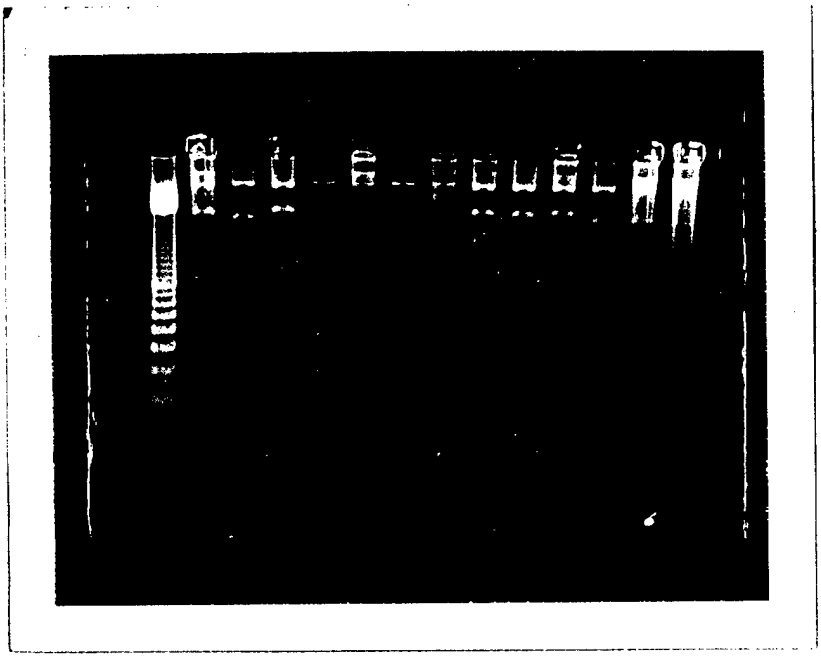


Figure 4. Transformed bacteria were cultured overnight,miniprepped, the recovered plasmid-insert constructs digested with BamH-1 and EcoR-1 and electrophoresed. Lane 1-100bp ladder. Lanes 2-4 -#1 construct. Lanes 5-8 -#4 construct. Lanes 9-11 - #1 construct. Lane 12 -#3 construct. Lanes 13-14 -#5 construct. As noted in the text, inserts are poorly, if at all visible. However, a plasmid-only control resulted in no transformants.

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Appendix I

I would like to respond, as asked, to reviewer comments from last year's annual report.

1) The reviewer is unclear as to the necessity of generating additional antibodies (which is where a lot of time is being spent), when we have OSW-2 available. I hope this is clear by now. One main goal is to determine the orientation of the 115kD subunit in the membrane by epitope mapping. This will require antibodies targeting internally and externally exposed domains, such that binding/uptake experiments can identify externally exposed epitopes. Subsequent labeling of fixed, permeabilized cells may further clarify our understanding of this subunit's positioning. In addition, OSW-2 does not work on Western blots, which renders it useless for some of the experiments I will be conducting. I apologize if I did not state this previously.
2) The reviewer is uncertain why the human cDNA was cloned, when the bovine cDNA was available. The short answer is that the library screening and cloning of the human cDNA was accomplished after this grant was awarded but before it went into effect. At the time, this work was part of a body of work I was doing simply to gain experience in molecular techniques. More substantively, we work with human breast cancer cells, and it is possible that antibodies raised against the bovine protein might not have recognized the human protein. Though we expected the two sequences to be very similar, without actual cloning and sequencing this could not be established. Further, as the 115kD subunit is so highly conserved across species, it is unlikely to be highly antigenic in the animal used for antibody production. As such, it might be expected that the most antigenic epitopes would be precisely those that are different and unique to the human sequence.
3) The reviewer requests data supporting the assertion that conditions of antibody uptake experiments (Task 3.1) have been worked out. This work was done while I was an indirect guest of a researcher in Germany and, as such, this work and these results are the property of that lab, and I am not comfortable requesting that these results be searched for and forwarded (it was a lab of 60-70 post-docs and graduate students). The experiments were very simple and straightforward, and have not been

reproduced here because of their simplicity and the difficulty we've experienced producing OSW-2 in our lab, as stated in the body of this report.